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Diagnostic Tools for Mycotic Pneumonia in large ruminant using autofluorescence character of fungi and detection of virulence gens of *Aspergillus fumigatus* using PCR

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Abstract: Mycotic pneumonia is a respiratory disease of farm animals caused by different species of the fungal genera. Lung affections in farm animals constitute serious problem that hinders animal production and may result in great losses in animal husbandry. The objectives of this study were to estimate the infection rate of Mycotic pneumonia in large ruminants, identify the incriminated fungal species and differentiate among them using conventional and Polymerase chain reaction (PCR) of internal transcribed spacer (ITS) along with detection of virulence gene (*Asphs*) involved in *A. fumigatus*. PCR along with culture results acted as gold standard methods for diagnosis of fungal cause. Also reported that autofluorescence considered as a rapid screening technique for diagnosis of fungal infections without the delay associated with special stains.
[Ashraf A. Abd El-Tawab, Eman M. El-diasty, Ahmed Abu Al-Zaid Fawaz and Emad E. El-Mougy **Diagnostic Tools for Mycotic Pneumonia in large ruminant using autofluorescence character of fungi and detection of virulence gens of *Aspergillus fumigatus* using PCR** *Nat Sci* 2021;19(3):44-52]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature 8. doi:10.7537/marsnsj190321.08.

Key words: *Aspergillus fumigatus*, ruminant, Virulence, autofluorescence, PCR,

1. Introduction

Respiratory disorders represent one of the most important causes of great economic losses among animals. Lung affections in farm animals constitute serious problem that hinders animal production and may result in great losses in animal husbandry. Pneumonia was a leading cause of lung condemnation **(Hafez *et al*, 1991 and Mahmoud *et al*., 2005)**. In ruminants, pneumonia is a complex condition that involves the interaction between the animal's immunity, physiological system, multiple pathogenic agents such as bacteria, virus and mycoplasmas, fungi and also environmental factors, whereas pour hygienic measures and climate disorders are the most predisposing factors to infection **(Jaja *et al.*, 2016).**

Aspergillus fumigatus is a saprophyte fungus which survives and grows over a large variety of organic remainder and whose most common ecological niche is on the ground. It is one of the most ubiquitous fungi, due to the ease of dispersion of its conidia. *Aspergillus fumigatus* causes a wide range of diseases that include allergic reactions, lung infection and systemic diseases (invasive aspergillosis) with high mortality rates **(Rementeria *et al*., 2005).**

Haemolysin is putative virulence factor thought to contribute to *A.fumigatus* pathogenesis. In particular, the secretion of haemolysin, followed by iron acquisition, facilitates hyphal invasion in disseminated Aspergillosis **(Odds, 1998).**

Fungal infection is a major health concern as the clinical features are not very. Lack of rapid diagnostic techniques results in delay in diagnosis, treatment and lead to death or slaughter of animal (economic losses). The fact that many pathogenic fungal organisms autofluoresce in hematoxylin and eosin stained sections under ultraviolet lightening, so this test is considered as a rapid screening technique for diagnosis of fungal infections without the delay associated with special stains (Rao *et al.*,2008).

Molecular approaches have been applied as an alternative assay replacing cumbersome and time-consuming microbiological and chemical methods for the detection and identification of mould **(Niessen, 2008)**. PCR-based methods for simultaneous detection of mould are useful tools to be used in diagnosis of diseases.

Molecular approaches have been developed to provide more rapid and accurate identification of

mould. The internal transcribed spaces 1 and 2 regions (ITS 1 and ITS

2) have been used extensively for molecular analysis of mould. The methods used are PCR, ITS fragment length polymorphism, restriction fragment length polymorphism, DNA probe hyperdezation and DNA sequences **(Logotheti et al., 2009).**

Hemolysin (Asp-HS). The protein is secreted into the environment and can kill cells that are in the proximity of the spore. The hemolysin, which enables the fungus to disrupt blood cells, contains negatively charged domains and can also be detected in infected animal. The hemolysin has toxic effects it seems not to be a main virulence factor but a compound that increases the effects of other toxic factors involved in pathogenicity **(Malicev *et al.*, 2007). T**he hemolysin produced by *A.fumigatus* (asp-hemolysin)promotes Aspergillosis and may also promote opportunistic infections, **(Theeb, *et al.*, 2013).**

The aim of this study was to isolate and identify the possible causative mould pathogen of such respiratory disease conditions as direct methods of their diagnosis. In addition to, assess the value of autofluorescence as a screening method for detecting mould and identification of isolates by PCR and detection of gene involved in *A. fumigatus* virulence

2. Material and Methods

2.1. *Collection of samples:*

A grand total of 50 lung samples of large ruminant animals (buffalo and cow 25 samples from of each) from abattoirs at Kalyobia Governorate. Samples collected from diseased animal suffering from respiratory manifestation. Tissue specimens from lungs were taken from the affected slaughtered animals (50 in number) the samples were visually examined for gross lesions. Each tissue sample was divided into two parts, one part was put in a sterile polyethylene bag in an ice box under aseptic conditions for mycotic isolation and the second part was immersed in 10% formalin saline for histopathological evaluation. Nasopharyngeal swabs were aseptically taken from the nasal clift and pharynx of the respiratory affected animals (50 in number) and transferred in icebox directly to the laboratory with minimum of delay. All samples were subjected to mycological examinations.

2.2. *Cultivation of sampling* (according to Anaissie *et al.*, 2003):

The nasopharyngeal swabs were directly cultured by streaking on the surface of two plates of Sabouraud dextrose agar media containing chloramphenicol 0.05mg/ml, inoculated plates were incubated at 25^0C for 5-7 days. While, the lung samples were immersed in 70% ethyl alcohol for 3 minutes to remove the external contamination and then the samples were opened and the contents were inoculated onto Sabouraud dextrose agar media.

2.3. *Identification of mould:*

All Isolated filamentous fungi were cultivated on Czapek yeast agar (CYA) , Malt extract agar (MEA) ,25% Glycerol nitrate agar (G25N) slopes and incubated for 5 days for their identification according to **(Klich, 2002; Klich and Pitt, 1988 and Frisvad and Samson, 2004).**

Macroscopical examination

Careful observation of the rate and pattern of growth, colour and texture of basal and surface mycelia. Also, the consistency of the surface , the reverse of the colony or diffusing into the surrounding medium were observed and recorded. The examination was carried out by using a magnifying hand lens.

Microscopical examination

One drop of lactophenol stain was added then covered by a clean cover slide (24x24mm) followed by gentle pressure to remove the excess of fluid and air bubbles as well as to depress the hyphae and other structures for facilitating microscopic examination. The prepared slides were then examined under microscope to characterize the measurements and morphological structures of the mould growth.

2.4. Determination of hemolytic activity:

Haemolysin activity was evaluated with a blood plate assay as described by **(Manns *et al.*, 1994).** Media were prepared by adding 10 ml fresh sheep blood to 100 ml SDA supplemented with glucose at a final concentration of 3% (w/v). A standard inoculum of both the test fungi isolates were deposited onto the medium. The plate was then incubated at 25^0C for 3 days.

2.5. Proteolytic Activity:

Glucose Yeast Extract Peptone Agar medium with 0.4% gelatin (pH 6.0) was used. 8% of gelatin solution in water was sterilized separately and added to GYP medium at the rate of 5mL per 100mL of medium. After incubation degradation of the gelatin was seen as clear zone around the colonies. The plate was then flooded with saturated aqueous ammonium sulphate, which resulted in formation of a precipitate. This made the agar opaque and enhanced the clear zone around the fungal colony **(Sunitha *et al.*, 2013).**

2.4. Tissue Preparation for autofluorescence (Stain H&E) Studies: Bancroft and Marilyn (2002):

Specimens from collected lungs were immediately taken from the slaughtered animals and immersed in 10 % formalin. The fixed specimens were trimmed, washed, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. The embedded samples were sectioned at 3-5 µm thickness, stained with H and E stain. Periodic

Acid Schiff stain (PAS) and Gomori Methenamine Silver (GMS) stains were used as special stains.

2.5. PCR Amplification

DNA extraction from six isolates (*Aspergillus fumigatus*) of the strains which had previously been identified macroscopical and microscopical examination were molecularly identified using the fungal primer, using Patho Gene-SpinTM as described by DNA/RNA Extraction kit iNtRON cat. No. 17154 Korea. The PCR primers used Forward PEX1 5' TATGTCTTCCCCTGCTCC -3 and Reverse PEX2 5' CTATGCCTGAGGGGCGAA -3 oligonucleotide primer used in PCR reactions were synthesized by Sigma Company, (Germany) at bp 250 **(Logotheti *et al.*, 2009)**. DNA samples were tested in 50 µl. reaction volumes in a 0.2 ml. eppendorf tube, containing 25 µl PCR Mix which was composed of (10X buffer, 10mM d NTPs mixture, Taq polymerase), 1 µl of each primers, 2 µl target DNA, complete to a final volume of 50 µ with sterile deionizer water. PCR amplification conditions for *Aspergillus fumigatus* strains were: 5 min initial denaturation step followed by 38 cycles at 94 °C for one min, 38 cycles at 59^{0}C for 1 min and a final extension step at 72 °C for 5 min. Amplification products were electrophoresed in agarose gels (1.5% w/v) (Agarose, Sigma, USA) and stained with ethidium bromide using Gene Ruler 100bp DNA Ladder for 10 min. for 30 sec and 35 cycles for 72 °C for 1min.

2.6. PCR protocol for amplification conditions of *A. fumigatus* virulent gene primer:

The primer sequences selected for virulent gene F-Asphs 5' TGGTACAAGGACGG TGACAA-3 and R-Asphs 5' GTCCCAGTGGACTCTTCCAA -3 which amplified a 185 bp fragment on genomic DNA(**Abad-Diaz-De-Cerio *et al.*,2013).** PCR master Mix:

MyFi™ Mix: Master Mix (2X) Bioline Company, Cat., No. BIO-25049, England. PCR protocol for amplification conditions of Asphs gene, initial denaturation 95^{0}C for 10 min one cycle; denaturation 95^{0}C for 1 min 32 cycles; annealing 60 ^{0}C for 1min 32 cycles; Extension 72 ^{0}C for 1min 32 cycles and final Extension 72 °C for 10 min one cycle. PCR products were analysed for the presence of specific fragments of the expected length in a 1.5 % agarose gel electrophoresis stained with Ethidium bromide. DNA sequence polymorphisms. DNA fragment purification Kit. The amplified products were purified using Gene JET PCR purification kit (USA) and were sequenced by colour Company, Egypt .All the strains were sequenced in both directions. Sequences were analyzed and aligned by Clustal method using the program DNAstar (Laser-gene, Wisconsin, USA).Sequencing was performed by sequencing to the PCR product on GATC Company by use ABI 3730x1 DNA sequences by using forward and reverse primers. Sequences were deposited at gene bank and phylogenetic analysis was done by MEGA X (Kumar et al., 2018), while sequence divergence and identity percent by DNA star (**Felsenstein, 1985; Jones *et al.*, 1992** and **Kumar *et al.*, 2018**).

2.7. Antifungal susceptibility testing

The isolates were subcultured on Sabouraud Dextrose Agar (SDA) and incubated at 25°C for 5 days. In vitro the sensitivity of the isolates to antimicrobials was determined according to standards of **National Committee for Clinical Laboratory (NCCLS, 2002)**. Spores from pure culture of *Aspergillus fumigatus* was mixed well with 9 ml of sodium chloride solution then spreading over the surface of SDA plate then suction the excess fluid. Six antifungal discs (Nystatin 100µg/ml, Clotrimazole 10 mcg, Itraconazole 10µg/ml, Voriconazole 200 mg, Amphotericin B100µg/ml and Fluconazole 10µg/ml) were spread on the surface of inoculated plates. Plates were incubated at 25^{0}C for 5 days. The diameter of inhibition zone of each disc was measured (mm) and judged.

3- Results and Discussion

Aspergillosis range from allergies to invasive Aspergillosis (IA), standing out because of their seriousness, Allergic bronchopulmonary aspergillosis, aspergilloma (ABPA) and several forms of invasive Aspergillosis such as invasive pulmonary aspergillosis (IPA) or chronic necrotizing aspergillosis (CNA). These infections are mainly acquired by inhalation, being lungs the primary focus and in less proportion paranasal sinus. Allergic bronchopulmonary aspergillosis is a hypersensitivity pulmonary disease due to an environmental exposure to *A. fumigatus* allergens. The fungus grows saprophytically colonizing bronchial tube and causing a persistent inflammatory response by immunoglobulin E (IgE) which leads to bronchial obstruction **(Zmeili and Soubani, 2007).** In affected cattle, infections with Aspergillus may be asymptomatic. In respiratory aspergillosis, respiratory symptoms such as coughing, dyspnoea and haemoptysis may be apparent. In some cattle, this can be rapidly fatal as dissemination of spores occurs through the pulmonary circulation.

The results recorded in **Table (1)** showed that a total of 100 lung samples and nasal swabs (25 from each) of buffalo and cow. Nine (36%) and ten (40%) samples from buffalo and cow lung samples were positive for mycotic infection, respectively. While in case of nasal swab twelve (48%) and fifteen (60%) samples from buffalo and cow were positive for

mycotic infection. The samples of lung and nasal swab collected from cow more infected with fungi than samples collected from buffalo. This result similar to those obtained by **Chihaya *et al.* (1991),** and **Radostits *et al.* (2007)** reported that the mycotic pneumonia is uncommon in farm animals but a high prevalence can be expected in calves and lambs kept in intensive housing units. **El- Metwally *et al.*, (2011)** examined a total number of 200 calves' lung tissues which collected from Giza abattoirs, Egypt during 8-months and found that the total mycological isolations were 116 cases (58%). While, **Shawky *et al.*, (2014)** reported that 25% were positive for presence of fungi isolated from buffalo calves. While, moulds were isolated from 15 cases in single and mixed form.

Results achieved in **Table (2) and Fig. (1)** declared that Aspergillus *spp.*, Penicillium and Mucor species. The most prevalent species in the examined samples were Aspergillus spp. and *Aspergillus fumigatus* was the most isolated Aspergillus spp. followed by Penicillium and Mucor species. The results of Aspergillus species isolation revealed that the *A. fumigatus* was recovered from lung tissue and nasal swab of buffalo and lung tissue and nasal swab of cow 4 (44.4%), 6 (60%), 6(50%) and 7 (46.7%), respectively. Whereas, the other species (*A.flavus* and *A.niger*) that isolated from lung and nasal examined samples were recovered in 11.1%, 20%, 8.3%, 20%, 11.1%, 20%, 16.7 and 20 % ,respectively. The fungus of Penicillium spp. was recovered from 22.2%, 10%, 8.3% and 6.7 % of lung tissues and nasal swabs samples. Mycotic infection is mainly caused by inhalation of spores, which can lead to and mortality in animals **(Kuehn, 2013). Shawky *et al.*, (2014)** reported that the haemolymphtic dissemination. *Aspergillus species, are* identified as the main causative agents of mycotic pneumonia. They cause significant economic losses, morbidity isolated mould species from single and mixed infection were 2.5 and 6.3% for *A.fumigatus*, 1.3 and 3.8% for *A.niger*, 0% and 1.3% for Penicillium. While 3.8 and 0% for Mucor .The recovery of fungal contamination in association of respiratory affection of ruminants were previously reported by (**Whittaker and Hogan, 2010).**

Table (1): Prevalence of pneumonia in examined samples of buffalo and cows based on culture growth

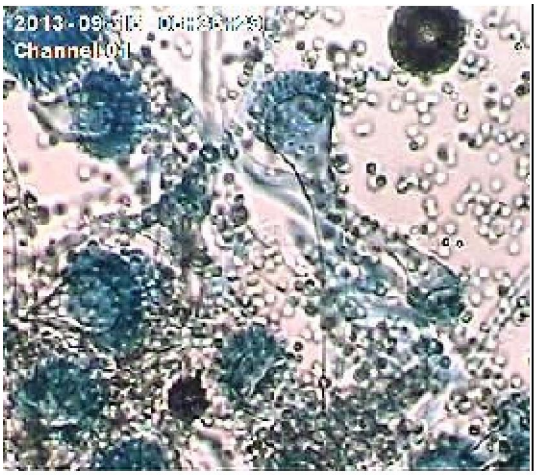
Animal	Lung			Nasal swabs		
	No. of samples	+ve	%	No. of samples	+ve	%
Buffalo	**25**	**9**	**36**	**25**	**12**	**48**
Cow	**25**	**10**	**40**	**25**	**15**	**60**
Total	**50**	**19**	**38**	**50**	**27**	**54**

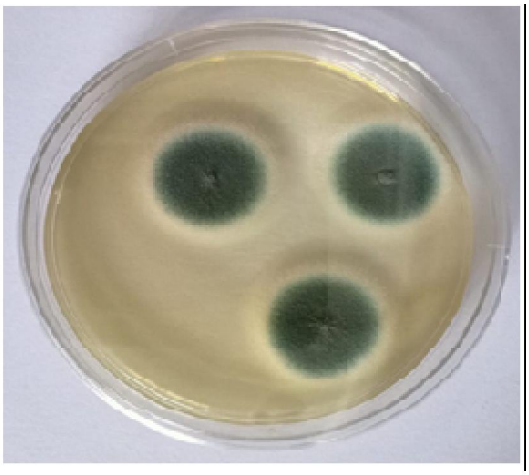
Table (2): Prevalence of isolated mould species from Lung and Nasal swabs examined samples

Isolated species	Buffalo				Cow			
	Lung tissue		Nasal swab		Lung tissue		Nasal swab	
	No	%	No	%	No	%	No	%
mould species								
A.fumigatus	**4**	**44.4**	**6**	**60**	**6**	**50**	**7**	**46.7**
A.flavus	**1**	**11.1**	**2**	**20**	**1**	**8.3**	**3**	**20**
A.niger	**1**	**11.1**	**2**	**20**	**2**	**16.7**	**3**	**20**
Penicillium	**2**	**22.2**	**1**	**10**	**1**	**8.3**	**1**	**6.7**
Mucor	**1**	**11.1**	**1**	**10**	**0**	**0**	**1**	**6.7**

Table (3): Prevalence of pneumonia in the lungs of **Buffalo** *and* **Cow** *based on Autofluorescence examination*

Animal	Lung			
	No. of samples		+ve	%
Buffalo	**9**	**7**	**77.8**	
Cow	**10**		**10**	**100**





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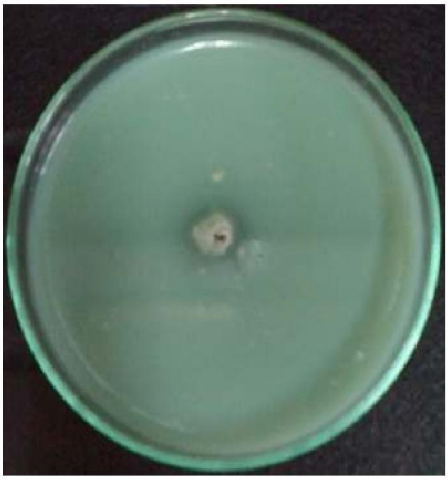

Fig. (2): left figure haemolytic activity of *A.fumigatus* on blood plate assay, right figure Proteolytic activity in *A.fumigatus* on yeast extract peptone agar plate, agar opaque and enhanced the clear zone around the *A.fumigatus* colony.

Many pathogenic fungi have been shown to exhibit fluorescence when H&E stained tissue sections are examined under a fluorescent microscope, and it has been suggested that the technique is useful in the identification of deep fungal organisms in tissue. Also reported that autofluorescence considered as a rapid screening technique for diagnosis of fungal infections without the delay associated with special stains **(Rao *et al.*, 2008)**. The two autofluorescent fungal species

identified were *A.fumigatus* **(Fig. 3)** within lung tissues and they exhibited strong enough fluorescence that the technique could be helpful. The architectural detail of bright green-to- yellow green autofluorescence.

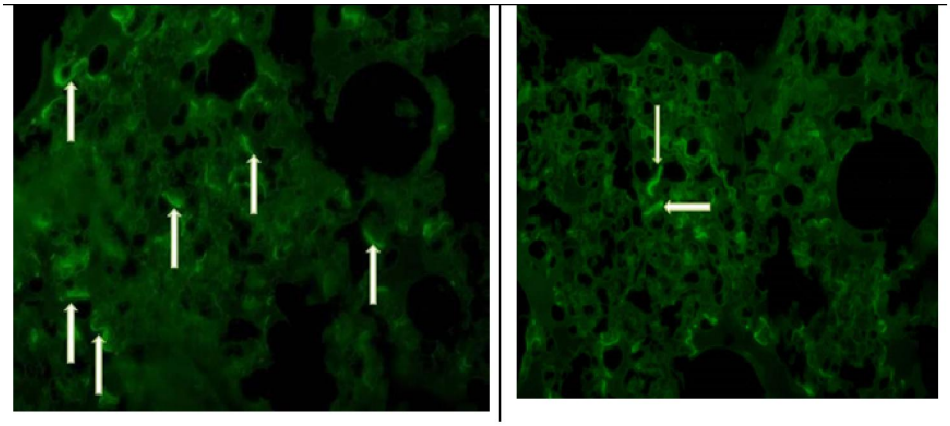

Fig. (3): *A.fum*

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Aspergillus se
and *Nidulante*. The most common species causing invasive disease is *A. fumigatus* in the section *Fumigati*. Molecular diagnostic approaches have revealed that there are at least 11 species that could easily be confused with *A. fumigatus* based on their conidial morphology. Besides *A. fumigatus,* the sister species *Aspergillus udagawae* (*Neosartorya udagawae*), *Aspergillus pseudofischeri* (*Neosartorya pseudofischeri*), *Aspergillus lentulus, Aspergillus felis, Aspergillus hiratsukae (Neosartorya hiratsukae*), *Aspergillus fischeranus (Neosartorya fischeri*), *Aspergillus viridinutans,*

Aspergillus fumisynnematus, Aspergillus fumigatiaffinis, Aspergillus novofumigatus, and *Aspergillus laciniosa (Neosartorya laciniosa*) have been reported sporadically in cases of aspergillosis.

In this study, the PCR primer pair was used

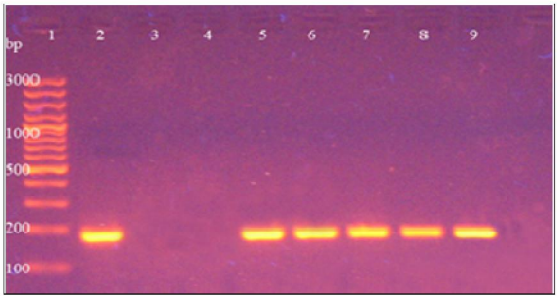

Fig. (4):1.5% of agarose gel electrophoresis of PCR product of (Asphs gene) virulent gene from representative clinical isolates of *A.fumigatus*. Lane 1: 100 bp ladder, Lane 2: control positive, Lane 3: control Negative and Lanes 4-9: Samples of *A. fumigatus.*

Of the strain of *A. fumigatus* studied, virulent gene Asphs could be amplified from six strains. The virulent gene Asphs of this *A. fumigatus* strain is readily distinguished from those of the other species by the 12 base insertion (TGGTCTGGCTTT) at nucleotides 113–125, and their distinctive nucleotide changes (T,C,T,G) at the following positions:-10-77-117 and 202 **(Figure 5).**

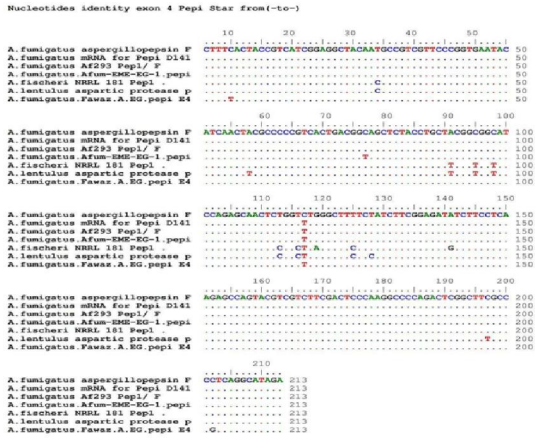

Figure (5): Multiple alignment of nucleotide sequence of *A. fumigatus* Fawaz. A.EG. pepi E4 *Asphs* gene compared to published sequence

A detailed comparison of virulent gene Asphs gene sequences demonstrated that certain base variations can be used to differentiate *A.fumigatus.Afum-EME-EG- 1.pepi; A. fischeri NRRL 181 Pep1.; A. lentulus aspartic protease p.* A differences comprise transitions at positions (31-15-25-32-33-38-39-42-47-66-68). Some of these variations result in amino acid changes **figure (6).** Phylogenetic trees were generated based on the virulent gene Asphs gene and the predicted toxin sequences. Phylogenetic analysis showed that *A. fumigatus. Fawaz. A. EG. pepi E4* (Acess no: MW546778) was isolated but in the same branches, from *A.fumigatus mRNA for Pepi D141*, but in different branches, away from *A.fumigatus.Afum-EME-EG-1.pepi;A. fischeri NRRL 181 Pep1* and *A. lentulus aspartic protease p* **(figure 8)**.

Nucleotide analysis in **figure (7)** showed greater than 98.6 % similarity between the sequenced *A. fumigatus. Fawaz. A. EG. pepi E4* strain and the other related six strains in the Gene Bank.

The one sequenced strain (*A.fumigatus*) and three isolates (*A.flavus, A.niger* and Penicillium sp.) were tested for antifungal sensitivity against five antifungal discs (Nystatin 100µg/ml, Clotrimazole 10 mcg, Itraconazole 10µg/ml, Voriconazole 200mg and Amphotericin B100µg/ml) as shown in table (4) Nystatin, Clotrimazole, Itraconazole and Voriconazole were the best and most effective antifungal while Amphotericin B has no effect on *A.fumigatus and A.flavus* the tested strains. We agree with **(Shawky, *et al.*, 2014)** in the effect of Voriconazole as they determined that it has no effect on *A.niger*, while Amphotericin B as they determined that it has effect on *A.fumigatus* and *A.niger*. **El-sebaey and Youssef (2019)** recorded that Terbinafine and Itraconazole effectiveness on *A. fumigatus* more than fluconazole and *A. fumigatus* showed resistance to griseofulvin. It is important that the increased use of antifungal agents has resulted in the development of resistance to these drugs. Like these of other living organisms, fungal cells may become resistant to toxic compounds. Many authors reported that increase in the resistance among fungi may be a worldwide problem and fungal pneumonia will become an increasing problem due to extensive use of non specific antibiotics for the treatment of pneumonia. dark turquoise (blue green).

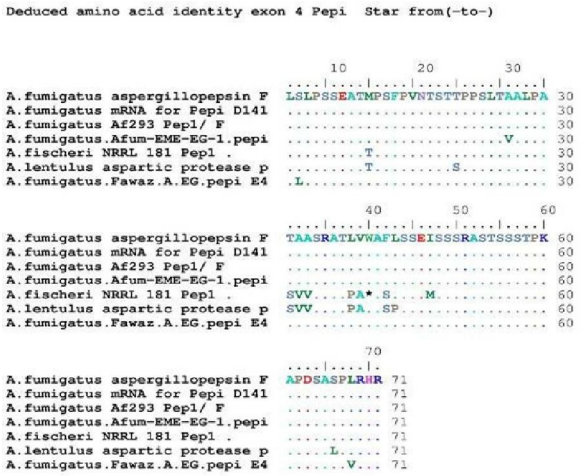

Figure (6): Multiple alignment of deduced amino acid sequence of *A. fumigatus* Fawaz. A.EG. pepi E4 *Asphs* gene compared to published sequence.

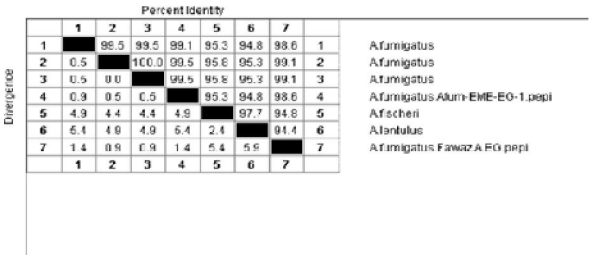

Fig. (7): Percentage identity of *A. fumigatus* Fawaz. A.EG. pepi E4 *Asphs* gene sequences in segment of exon 4 partial compared to published sequences.

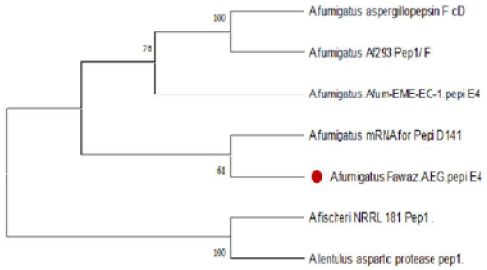

Fig. (8): Phylogenetic tree of nucleotide sequences of *A. fumigatus* Fawaz. A.EG. *Asphs* virulent gene compared to published sequences

Table (4): Antifungal susceptibility testing of fungal isolates

Isolates	NS100	CC10	IT10	VRG	AP100
A.fumigatus	I	S	S	S	R
A.flavus	S	I	I	S	R
A.niger	S	S	I	I	S
Penicillium sp.	S	S	S	S	S

S= Susceptible I= Intermediate R=Resistance

5. Conclusion:

It could be concluded from the aforementioned result that the important agent causing pneumonia in cattle *A.fumigatus*, *A.flavus* and *A.niger* were isolated from pulmonary tissues and virulence factor of isolates revealed that maximum extracellular protease activity and haemolytic activity were observed in Aspergillus species. The technique of autofluorescence is a simple and quick procedure, which can be done on routinely prepared H and E-stained slide using fluorescent microscope without addition of any immunoreagents. Its high sensitivity and specificity make it an ideal screening method.

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3/16/2021